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DIFFERENTIAL RADIOPROTECTION BY GLUTATHIONE OF TWO GROWTH FUNCTIONS IN THE HYDROID *CAMPANULARIA FLEXUOSA*

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The phenomena of growth, development, and regeneration in cnidaria have been previously studied by the use of x-irradiation. In most cases, x-irradiation leads to deleterious changes in these organisms. Puckett (1935) reported that the regeneration of amputated hydranths of the hydroid *Pennaria tiarella* was prevented by an x-ray dose of 10,000 roentgens. Daniel and Park (1951, 1953) produced considerable damage to the tentacles of the common brown hydra either by x-irradiating the animals with 9600 roentgens, or by incubating nonirradiated animals in irradiated saline solution. Damage was considerably reduced if the animals were irradiated in 10^{-5} *M* glutathione-saline solution, or if 10^{-5} *M* glutathione was added to the saline solution before the solution was irradiated, and before nonirradiated animals were added to it.

On the other hand, Brock and Strehler (1963), and Strehler (1964), employing x-ray doses of from 500 to 210,000 roentgens on ten-day-old colonies of *Campanularia flexuosa*, reported an increase with increasing dose in the life span of the normally cyclically regressing hydranths of this species. Wermuth and Barnes (1967) in a preliminary report presented evidence for an increase in the growth rate of new, nonirradiated stolons of *Campanularia flexuosa* when the starting material of the new colonies received an x-ray dose of 81,000 roentgens on the fifth day after the initiation of the colonies. The present study extends these observations to include a study of the effects of glutathione incubation on the previously reported increase in new stolon growth rate. New hydranth production under conditions of irradiation, and the effects of glutathione incubation followed by x-irradiation were also observed. Finally a hypothesis concerning stolon growth control is proposed.

Materials and Methods

Stock colonies were originally obtained from Dr. P. Sears Crowell, and Mr. Robert Suddith, Department of Zoology, Indiana University, Bloomington, who had collected them near Woods Hole, Massachusetts. The stock colony organisms have been vegetatively propagated as a clone for the past two years. Cultures of test organisms were established according to the method of Crowell (1953). The starting material for a new colony, consisting of a hydranth-bearing

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stem (upright) from a stock colony, was slipped under a cotton thread previously tied around a microscope slide. Within a day, the cut end of the upright repaired itself and new stolon growth began from the cut end. The term "starter" designates the upright and associated hydranths originally placed under the cotton thread; the term "stolon" designates the minimal amount of the starter upright on the side of the thread opposite the starter, and the new material which grows out from the cut end (Fig. 1).

The slides with attached colonies were placed in glass staining racks. The colonies of the first four series were placed in glass staining dishes on a moving table. The sea water in the staining dishes was changed weekly. All subsequent colonies were placed in a flowing sea-water system. The temperature of all sets of colonies was maintained at 12° C. Colonies were fed once a day on *Artemia* (brine shrimp) nauplii just prior to examination. Each feeding hydranth was allowed to capture as many brine shrimp as possible. X-irradiation was delivered

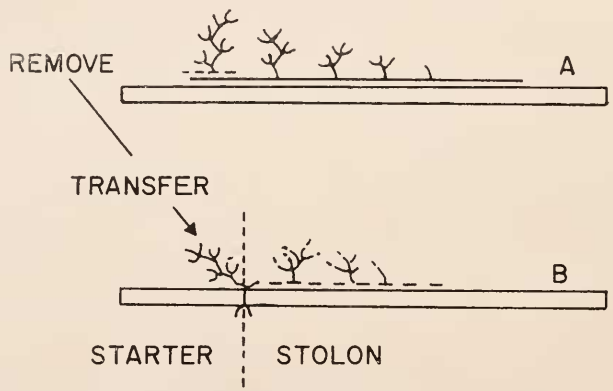

FIGURE 1. The method of starting new colonies of *Campanularia flexuosa* (after Crowell, 1953); A. Stock colony, B. new colony.

by a General Electric Maxitron Two-Fifty x-ray machine operated at 250 KVP and 15 milliamperes unfiltered. The distance from the x-ray source to the test colonies was 15 cm. Colonies to be irradiated were placed on a lead plate in an open Petri dish and then just covered with sea water. This Petri dish was placed on a larger, covered Petri dish filled with crushed ice. For differential irradiation, three one-eighth-inch thick lead plates were placed over the part of the colony to be protected. X-ray dosage as measured by a Victoreen r-meter at the position of the animals was 81,000 roentgens (8100 r/min for series 1 through 8, and 7600 r/min for series 9 through 18). Stray irradiation under the shielding was 40–50 r/min. Either whole colonies, starters only, or new stolons only were irradiated.

In those experiments involving glutathione incubation before irradiation, all colonies were placed in 10^{-5} *M* glutathione-sea water solutions in glass staining dishes. Glutathione-incubated colonies were irradiated in fresh sea water, except for the colonies of series 17. The pattern of irradiation, and glutathione incu-

bation and subsequent irradiation for all the colonies described in this paper are listed in Table I. The glutathione used in these experiments was obtained from Calbiochem (lot number 73398) as crystalline reduced glutathione.

The standard "t" test, as described by Edwards (1958) was used to test for the possible statistical significance of the difference between the average daily stolon lengths of paired series.

Results

Irradiation of growing stolons with 81,000 roentgens produces the following changes in the stolon. Three days after irradiation, the new stolon stops growing. By the fourth day, the normally bulbous-appearing stolon tip loses its rounded

Table I

Pattern of irradiation and glutathione incubation followed by irradiation

Series	Number of colonies	Part of colony irradiated, and day of irradiation	Glutathione incubation
1	7	None	None
2	9	Stolon only, day 5	None
3	8	Starter only, day 5	None
4	7	Whole colony, day 5	None
5	9	None	All colonies of these four series were incubated in a freshly prepared solution from day 4 to day 5.
6	9	Stolon only, day 5	
7	9	Starter only, day 5	
8	8	Whole colony, day 5	
9	6	None	None
10	10	Starter only, day 6	None
11	8	None	Prep. day 4, inc. days 4–6
12	9	Starter only, day 6	Prep. day 4, inc. days 4–6
13	9	None	Prep. day 3, inc. days 4–6
14	9	Starter only, day 6	Prep. day 3, inc. days 4–6
15	11	None	Prep. day 4, inc. 30 min day 4
16	11	Starter only, day 6	Prep. day 4, inc. 30 min day 4
17	11	None	Prep. day 6, inc. during irr.
18	9	Starter only, day 6	Prep. day 6, inc. during irr.

appearance and becomes square. Half-moon-shaped "dents" begin to appear in the sides (and presumably the top and bottom) of the coenosarc of the irradiated stolon. Eventually the dents enlarge so that the coenosarc is discontinuous. Within a week to ten days after the cessation of stolon growth, all cellular material has disappeared, presumably by autolytic processes, leaving the empty perisarc tubes undisturbed. In stolon-only irradiated colonies, new stolons may push into these hollow perisarc tubes from non-irradiated starters.

Irradiated uprights are unable to produce new hydranths. The hydranths of this species undergo a regression-regeneration cycle (Crowell, 1953). Beyond a certain developmental stage, an irradiated hydranth will continue to differentiate until it becomes a complete, normal feeding structure. An irradiated complete

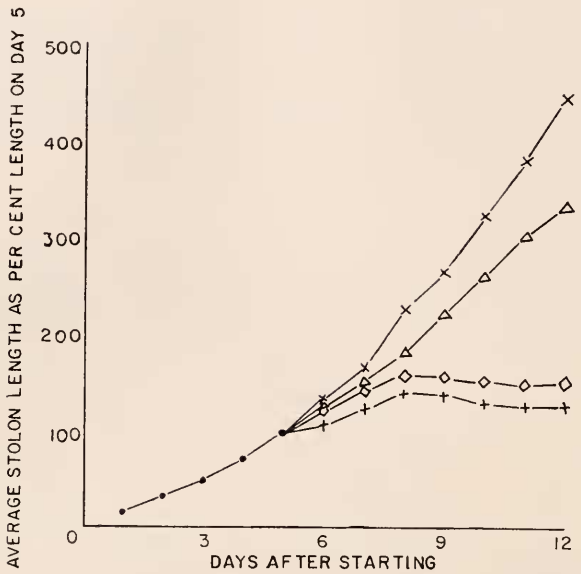

FIGURE 2. Average daily stolon lengths of colonies of *Campanularia flexuosa* as per cent of length on day irradiated (day 5) *vs* time. Colonies were not incubated in glutathione-sea water before irradiation. Symbols are: △, no irradiation (series 1); +, stolons only irradiated (series 2); X, starters only irradiated (series 3); ◇, whole colonies irradiated (series 4); ●, average of series 1 through 4 from day 1 through day 5.

hydranth will not regenerate after regressing, nor will an irradiated regressing or regressed hydranth regenerate a new hydranth. These irradiation-caused changes in stolons and hydranths are not reversed by incubation in glutathione-sea water solutions.

The results of x-irradiation on new stolon growth after the irradiation of new stolons only (series 2), starters only (series 3), and whole colonies (series 4) are presented graphically in Figure 2. The new stolons of starter-irradiated

TABLE II

Effects of x-irradiation, and glutathione incubation followed by x-irradiation on new stolon growth. Results of "t" tests (as "*P*" values)* for differences between average daily stolon lengths of paired series. For experimental situation of each series, see Table I.

Series	Day						
	1–5	6	7	8	9	10	11
1 and 3	—	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
2 and 4	—	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
5 and 7	—	<0.01	<0.02	<0.05	—	—	—
6 and 8	—	—	—	—	—	—	—

* (—) represents values of $P > 0.05$.

colonies (series 3) continue to grow after irradiation. Furthermore, they grow at a greater rate post-irradiation than do new stolons of control colonies (series 1). The new stolons of wholly irradiated colonies (series 4) achieve a greater rate of stolon growth than do the stolons of stolon-only irradiated colonies (series 2), at least for the first three days after irradiation. At this time, the irradiated stolons of the stolon-only irradiated colonies of series 2 and the irradiated stolons of the wholly irradiated colonies of series 4 stopped growing. Probability values for the significance of the differences between the average daily stolon lengths of series 1 and series 3, and series 2 and series 4 are presented in Table II. For days 6 through 11, the differences between the average daily values of stolon lengths are highly significant ($P < 0.01$) for series 1 and 3, and series 2 and 4.

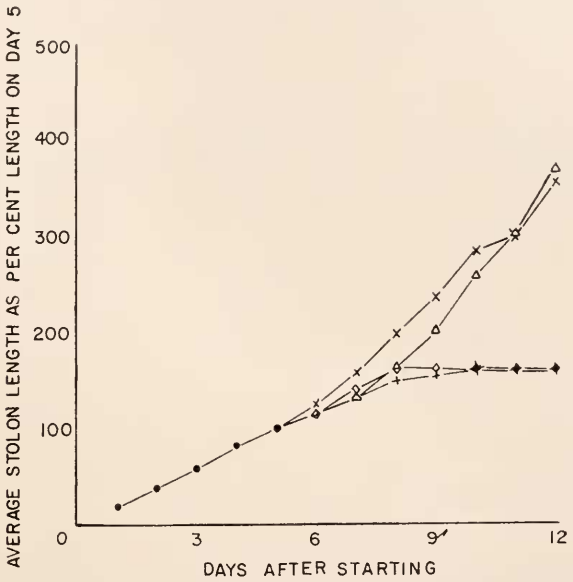

FIGURE 3. Average daily stolon lengths of colonies of *Campanularia flexuosa* as per cent of length on day irradiated (day 5) *vs* time. Colonies were incubated in fresh glutathione-sea water for 24 hours before irradiation. Symbols are: △, no irradiation (series 5); +, stolons only irradiated (series 6); X, starters only irradiated (series 7); ◇, whole colonies irradiated (series 8); ●, average of series 5 through 8 from day 1 through day 5.

The studies by Daniel and Park (1951, 1953) led us to test whether reduced glutathione in sea water would alter the radiation-induced changes in stolon growth and hydranth production described above. Accordingly, another four series of colonies (series 5 through 8) were irradiated in fresh sea water after they had been incubated in a freshly prepared 10^{-5} M reduced glutathione-sea water solution. A list of these experiments appears in Table I.

The results of the effects of x-irradiation at day 5 of new stolon growth of colonies which had been incubated in a freshly prepared glutathione-sea water solution from day 4 to day 5 are presented in Figure 3 and Table II. While there

are statistically significant differences between the average daily values of stolon lengths of series 5 (no irradiation) and series 7 (starters irradiated) for the first three days after irradiation, this significance is not shown for the differences on days 4 through 6 after irradiation. There are no statistically significant differences between the average stolon lengths of series 6 (stolons only irradiated) and series 8 (whole colonies irradiated).

A 10^{-4} *M* solution of reduced glutathione in sea water was analyzed for changes in concentration according to the method of Jocelyn (1967). The concentration of reduced glutathione was found to decrease to one-half of its original value in approximately 30 minutes. We therefore designed a pattern of glutathione incubations followed by irradiation to determine whether reduced glutathione *per se* had a radioprotective effect. These experiments (series 9 through 18) were limited to starter-irradiated and nonirradiated colonies. Irradiation was applied on day 6 after the initiation of new colonies. A list of these experiments appears in Table I.

TABLE III

Effects of glutathione incubation followed by x-irradiation on new stolon growth. Results of "t" tests (as '*P*' values)* for differences between average daily stolon lengths of paired series. For experimental situation of each series see Table I.

Series	Day						
	1–6	7	8	9	10	11	12
9 and 10	—	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
11 and 12	—	—	—	—	—	—	—
13 and 14	—	—	—	—	—	—	—
15 and 16	—	—	—	—	—	—	—
17 and 18	—	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

* (—) represents values of $P > 0.05$.

The results of the effects of x-irradiation applied to starters on new stolon growth after variable incubation in glutathione-sea water solutions are presented in Table III. The relative rate of new stolon growth of the nonincubated starter-irradiated colonies (series 10) is still significantly greater than the relative rate of new stolon growth of the nonincubated nonirradiated control (series 9). The pattern of these results is the same as that for series 1 and 3. In the remaining series of this set (series 11 through 18), statistically significant differences for average daily stolon lengths were found only for the paired series 17 and 18 (Table III). The colonies of these two series were incubated in a freshly prepared 10^{-5} *M* reduced glutathione-sea water solution during the course of irradiation on day 6 (series 18), or were incubated in glutathione-sea water for 10 min 40 sec on day 6 (series 17). The differences in average daily stolon growth values on nonirradiated and starter-irradiated colonies were statistically nonsignificant when colonies were incubated in fresh glutathione-sea water for 48 hours before irradiation (series 11 and 12); when colonies were incubated in 24-hour-old glutathione-sea water for 48 hours before irradiation (series 13 and 14); or when colonies were incubated for 30 minutes on day 4 in fresh glutathione-sea water.

Discussion

It would appear from the results of series 1 through 4, and series 9 and 10 that the rate of growth of new stolons of young colonies of *Campanularia flexuosa* is at least partially controlled by some part, or all of the upright used to initiate the colonies. The control seems to be of an inhibitory nature. Under certain conditions of irradiation of the starter upright, this inhibition of the rate of new stolon growth is suppressed resulting in an increased rate of new stolon growth. The evidence for the role of inhibition in the growth and regeneration of cnidarians has been well documented by Morgan (1901), Lund (1921), Rose (1957), and others.

The increased rate of stolon growth of starter-irradiated colonies disappears under certain conditions of incubation in 10^{-5} *M* reduced glutathione in sea water (Fig. 3, Table II and III). In these cases the differences in the relative rates of stolon growth of starter-irradiated colonies and their nonirradiated counterparts are not statistically significant. We interpret these experimental results as the radioprotection by some form of glutathione of an inhibitor system controlling stolon growth. An alternate hypothesis is that of radiosensitization by some form of glutathione. If the radiosensitization hypothesis is correct, one would expect the maximum average values of new stolon length achieved by the starter-irradiated colonies which have been glutathione incubated (series 12, 14, 16, and 18) to exceed the maximum average value of new stolon length achieved by the non-incubated starter-irradiated series (series 10). This is not the case.

It would seem that reduced glutathione is not the radioprotective substance, but rather that either some reaction product of reduced glutathione in sea water, or some metabolic product of glutathione is the radioprotective substance. This conclusion is derived from the following observations and assumptions. When colonies were incubated in a freshly prepared solution of reduced glutathione in sea water for 48 hours prior to irradiation (series 11 and 12), the concentration of reduced glutathione in the incubating medium would have been lowered to nearly zero by the end of the incubation period, assuming that the concentration of reduced glutathione decreases by one half in each half hour period. For series 13 and 14, "aging" the reduced glutathione in sea water lowers the concentration to about 5000 molecules per 250 ml of solution at the time the colonies were added to the solution for incubation. Considering the volume of the tissue of the colonies, and assuming that the reduced glutathione diffuses readily into the tissues of the colonies, it would seem that there was insufficient amounts of reduced glutathione present in the tissues to be radioprotective. Incubation of colonies for 30 minutes, 2 days prior to irradiation (series 15 and 16), and the resulting radioprotection afforded the proposed inhibitory system indicates that 30 minutes is sufficient time for the reduced glutathione or its sea-water reaction product to enter the tissues of the colonies. The 48 hour time lag between the end of the incubation period and the irradiation of the starters of the colonies suggests that some metabolic product of reduced glutathione is the radio-protective substance. If the preceding analysis is correct, it is not surprising that incubation in the reduced glutathione-sea water solution during the irradiation period affords no radioprotection of the stolon growth inhibitory system. Perhaps the rate of movement of reduced glutathione into the animals is too slow; perhaps

the tissue machinery which produces the radioprotective material is damaged by the irradiation itself, or the machinery is too slow to provide sufficient radioprotective substance in so short a time.

Incubation of colonies for 24 hours prior to irradiation does not protect the growing stolon from direct irradiation damage (series 6 and 8). The time course and events (cessation of stolon growth, squaring of the stolon tip, appearance of dents in the coenosarc, the dissolution of the coenosarc) involved in the death and destruction of the irradiated growing stolon are the same for these two series as for series 2 and 4 which were not incubated prior to irradiation. It is tempting to hypothesize that there is a specific stolon growth inhibitory substance produced by the starter, and that the production of this inhibitor substance can be decreased or eliminated by x-irradiation of the starter. The reversal of the increased rate of stolon growth by x-irradiation followed by glutathione incubation does nothing to convince us that we are dealing with a stolon growth inhibitory system rather than a stolon growth inhibitory substance. We do not, however, have any direct biochemical evidence that such an inhibitory substance exists.

We conclude that in normally growing colonies of *Campanularia flexuosa*, stolon growth consists of two functions: (1) a general growth system and (2) an inhibitory system superimposed on the first. Both functions are affected by irradiation but only the second function is protected by glutathione.

Summary

1. Five- and six-day-old colonies of *Campanularia flexuosa* were subjected to x-irradiation with and without variable incubation in reduced glutathione-sea water solutions.

2. X-irradiation doses of 81,000 roentgens alone stop hydranth production and stolon growth, the latter after three days.

3. Irradiation of starters resulted in an increased rate of new stolon growth over controls.

4. Under certain conditions of incubation in glutathione-sea water prior to irradiation, there was no increase in new stolon growth when starters were irradiated.

5. An hypothesis of stolon growth control is proposed.

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